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RAPID COMMUNICATION

Protein Tyrosine Kinase Activity during Egg Gastrulation in the Sea Urchin Embryo

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Fertilization triggers a series of preprogrammed events functioning to activate egg metabolism, incorporate the paternal genome, and initiate development. The activity of protein tyrosine kinases during egg activation is required for several steps leading up to the first cell division. We now present evidence for an additional protein tyrosine kinase-mediated event that occurs between 30 and 45 min after insemination and is not required until gastrulation, which occurs over 24 hr later. Eggs treated with protein tyrosine kinase inhibitors within this window of time cleaved and formed normal blastulae but could not gastrulate or undergo further development to the pluteus stage. These findings provide the first evidence that some of the control mechanisms used in later development are established during a brief period of time in the fertilized egg and require the action of one or more protein tyrosine kinases. © 1995 Academic Press, Inc.

INTRODUCTION

Fertilization results in the activation of various protein kinases including one or more protein tyrosine kinases (PTKs) (Satoh and Garbers, 1985; Peaucellier *et al.,* 1988; Ciapa and Epel, 1991; Abassi and Foltz, 1994). Studies using a variety of protein tyrosine kinase inhibitors have demonstrated a requirement for PTK activity during the later steps of the egg activation process. Events such as pronuclear migration and fusion, initiation of DNA synthesis, and mitosis were reversibly blocked by PTK inhibitors (Moore and Kinsey, 1995) and effects on sperm aster formation have also been demonstrated (Wright and Schatten, 1995). In order to more carefully define the requirement for PTK activity in these steps, we initiated a series of experiments in which fertilized eggs (zygotes) were treated with various PTK inhibitors for 15-min windows of time during the period between fertilization and the first cell division. These treatments had little effect on development up to the mesenchyme blastula stage. However, treatment of zygotes for a 15-min period between 30 and 60 min postinsemination irreversibly blocked the gastrulation process. These results demonstrate that the action of PTKs during egg activation can have far reaching effects on specific aspects of embryonic development.

MATERIALS AND METHODS

To evaluate the requirement for PTK activity at different points during the egg activation process, eggs from *Strongylocentrotus purpuratus* or *Lytechinus pictus* were fertilized and then incubated with 0.1% DMSO[2] (control) or with genistein (70 μM), erbstatin analog (20 μM), or tyrphostin B42 (80 μM) for 15-min periods after which the inhibitor was washed out and the embryos were cultured at 16°C in artificial seawater buffered with 5 mM Taps, at pH 8.3 (Taps-SW), as described (Moore and Kinsey, 1995). The progress of the embryos was monitored by light microscopy, and embryos were scored as having formed a blastula if clear evidence of a blastocoel was present. If an archenteron had

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[2] Abbreviations used: DMSO, dimethyl sulfoxide; Taps, 3-[tris-(hydroxymethyl)methyl] aminopropanesulfonic acid; PTK, protein tyrosine kinase.

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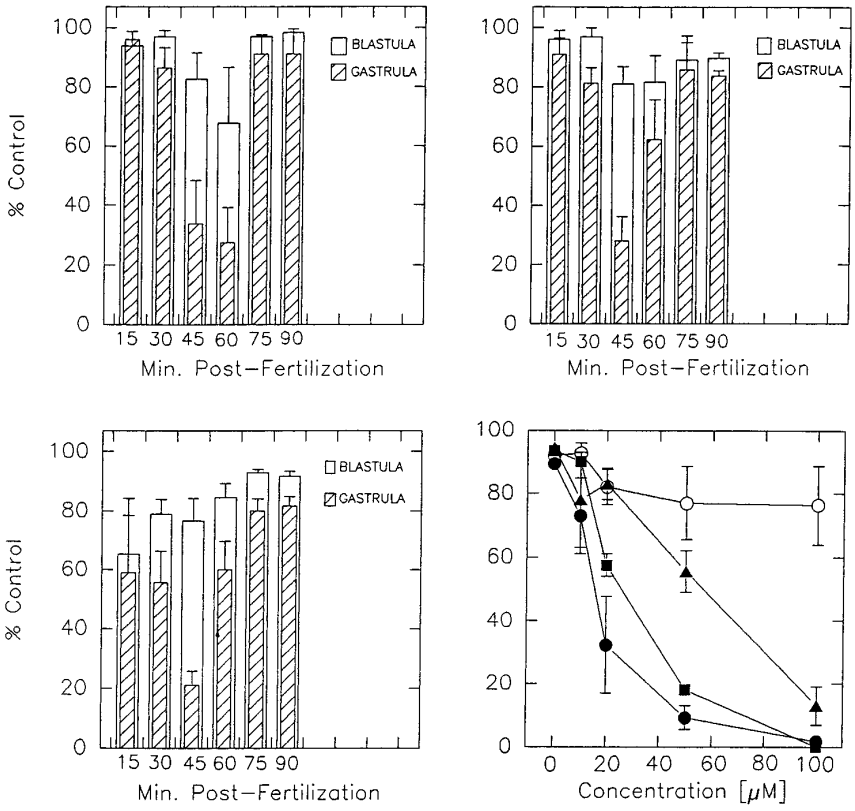

FIG. 1. Effect of PTK inhibitor treatment on later development. Eggs from *S. purpuratus* were acid dejellied and suspended in artificial seawater buffered with 5 m*M* TAPS (Taps-SW) and maintained at 16°C as a 0.1% suspension. The eggs were fertilized and then incubated with 70 μM genistein (top, left), 20 μM erbstatin analog (bottom, left), 80 μM tyrphostin B42 (done with *L. pictus*) (top, right) at different points postinsemination. After a 15-min incubation, the inhibitor was removed and the eggs were washed twice with 50 vol of Taps-SW. Development was monitored by microscopic examination over the next 48 hr. Groups of 100 embryos were counted at 36 hr and scored as having successfully undergone blastula formation (open bars) or as having successfully undergone gastrulation (cross-hatched bars). The results are expressed relative to the value in the control cultures treated with DMSO solvent only, in which over 90% typically gastrulated. Values represent the average of three experiments ±SE. One-way analysis of variance indicated that the percentage gastrula at 30–45 and 45–60 min differed from the other time points with an overall P value < 0.0001. The effect of inhibitor concentration was examined by treating zygotes between 30 and 45 min postinsemination with different concentrations of genistein (■), erbstatin analog (●), tyrphostin B42 (▲), or daidzein (○), and then quantitating the effect on subsequent gastrulation (bottom, right).

formed, embryos were scored as gastrula stage. The above inhibitors were purchased from Calbiochem.

RESULTS AND DISCUSSION

Zygotes treated with PTK inhibitors during the first 30 min of development experienced a delay in reaching the first mitosis (not shown) but recovered and cleaved normally forming normal appearing gastrulae by 36–40 hr (Fig. 1). However, zygotes treated between 30 and 45 min or between 45 and 60 min after insemination exhibited a markedly reduced ability to gastrulate. Further culture to 60 hr resulted in no further development of the treated embryos, indicating that gastrulation had been prevented rather than delayed. This period of sensitivity was transient, however, and zygotes treated at 60 min or later developed normally.

The effect of PTK inhibitor concentration in preventing gastrulation following treatment between 30 and 45 min postinsemination is shown in Fig. 1 (bottom, right). Optimum concentrations of erbstatin analog (20 μM), genistein (70 μM), and tryphostin B42 (80 μM) are somewhat higher than those used in our previous study (Moore and Kinsey, 1995), but here the inhibitors were applied only for 15 min vs 60 to 120 min in the earlier study. The inhibitor concentrations used here are within the range shown to inhibit PTK activity in homogenates of treated eggs, with little effect on protein kinase C or cAMP-dependent kinase activity (Moore and Kinsey, 1995). Nonetheless, erbstatin did have some irreversible inhibitory effect on later development as evidenced by lower rates of blastula formation and

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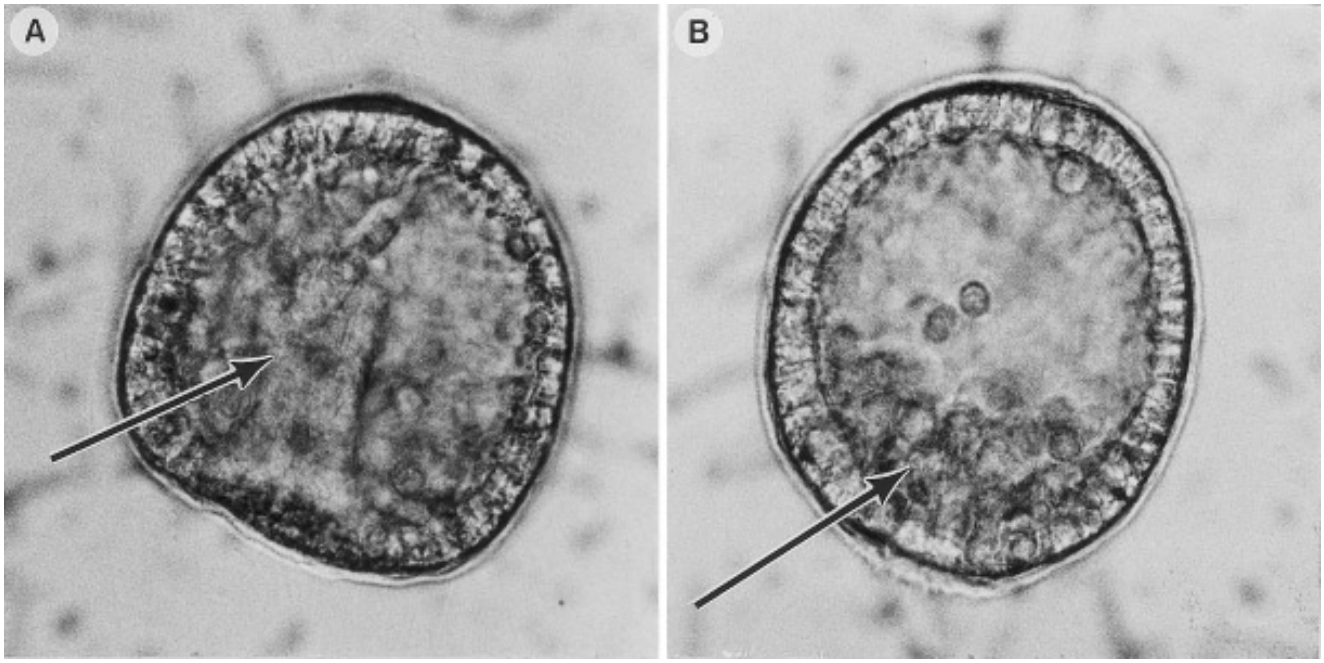

FIG. 2. Morphology of embryos derived from genistein-treated eggs. Samples of embryos from control (A) and genistein-treated (B) eggs were examined microscopically at 40 hr after fertilization. The control embryos (A) exhibit a normal archenteron (arrow) with associated mesenchyme cells visible inside the blastocoel. Genistein-treated embryos (B) did not form an archenteron although the mesenchyme cells have ingressed and accumulated at the site where the archenteron should form (arrow). Original magnification approximately 700×.

gastrulation. This may represent a nonspecific effect or possibly less reversible inhibition of PTK activity. The less potent member of the tyrphostin family, daidzein, had very little effect on development at all concentrations tested. In addition, it is significant that genistein, erbstatin analog, and tyrphostin B42 had the same effect on development even though genistein inhibits PTK activity through interaction with the ATP binding site of the kinase, while erbstatin and the tyrphostins inhibit kinase activity by interacting with the peptide binding site of the kinase (Ogawara *et al.*, 1986; Umezawa and Imoto, 1991). Since these inhibitors act through different mechanisms, it is unlikely that they would all have the same nonspecific effects. In addition to the PTK inhibitors, we tested the effect of H7 (10 μM), an inhibitor of cAMP-dependent kinase and protein kinase C, as well as okadaic acid (1 μM), a phosphatase inhibitor. These inhibitors had no effect on later development.

Microscopic examination of control embryos at 36 to 40 hr after fertilization revealed that they had gastrulated properly as indicated by the presence of an archenteron extending from the vegetal plate (Fig. 2A). Embryos derived from zygotes treated with genistein 30 to 45 min postinsemination had formed normal blastulae and exhibited mesenchyme cells at the vegetal plate (Fig. 2B). However, the embryos derived from treated zygotes had not gastrulated as evidenced by the lack of an archenteron. This suggests that the defect is probably related to the morphogenetic movements involved with invagination of the vegetal plate rather than to the differentiation and ingression of the mesenchyme cells. While the embryos derived from DMSO (control)-treated zygotes developed normal pluteii, no evidence of spicule formation was detected in the gastrulation-defective embryos and they did not elongate and flatten as would occur during normal development to the pluteus stage.

The results of this study demonstrate that inhibition of PTK activity between 30 and 60 min postinsemination has effects on embryonic development that do not become apparent until gastrulation begins. Identical treatment delivered before or after this ''window'' of sensitivity did not inhibit gastrulation, arguing against simple toxic effects as does the fact that other protein kinase and phosphatase inhibitors did not affect gastrulation. The implication of these results is that tyrosine phosphorylation of proteins in the fertilized egg is required for a function that occurs over 24 hr later in development. The window of sensitivity occurs just after pronuclear fusion and corresponds with the first cycle of DNA synthesis which begins at 30 min postinsemination and is largely completed by 40 min (Hinegardner *et al.*, 1964). It seems unlikely that our inhibitors affected DNA synthesis since development through the blastula stage was normal; however, it is possible that initial transcription of zygotic genes could be prevented. PTK activity is known to activate transcription factors in other systems and it is possible that transcription of one or more genes in

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the zygote is necessary for gastrulation. In any case, it is clear that the egg activation process involves more than just the metabolic activation of the egg. Protein tyrosine kinase-mediated events occurring during a short period during egg activation have far reaching effects on the development of the embryo and identification of the kinases and their substrates should provide further insight into this novel process.

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